Chapter 1

General introduction
ADVERSE DRUG REACTIONS

Adverse drug reactions (ADRs) have been a leading cause of attrition of drugs during development and clinical use. Toxicity has been estimated to be responsible for one-third of all drug attritions in the clinic, and toxicity and safety issues accounts for over half the failures of drug candidates in preclinical development (1). The majority of these ADR (about 75%) have been estimated to be of so-called “Type A”: dose-dependent adverse reactions that may be predicted based on the pharmacological profile of the drug candidate (2). These unwanted effects can either be primary, related to the drug action with the intended on-target, or secondary by interaction of the drug with an off-target. However, some ADRs have proven to be unpredictable and lack a clear dose-relationship. These reactions have been designated “Type B” (Bizarre) or “Type D” (Delayed) ADRs and are generally referred to as Idiosyncratic Drug Reactions (IDRs). Although these IDRs occur relatively infrequently and only in a small subpopulation of susceptible patients, they are often life threatening (3). Virtually any organ may be affected, but skin, liver and blood cells seem most vulnerable to IDRs (4).

Involvement of chemically reactive metabolites in ADRs and IDRs

For several drugs causing ADRs, including rare IDRs, bioactivation to chemically reactive metabolites (CRM) is believed to play an important role, as is depicted in figure 1 (4, 5). Such CRM may interact with cellular macromolecules (e.g. proteins or DNA) thereby inhibiting key functions within the cell leading to e.g. cytotoxicity, mutagenicity or genotoxicity. Alternatively, the CRM may indirectly affect signalling pathways, initiating a complex cascade of cellular responses leading to an ADR (6). Mechanisms underlying IDRs are even more complex and difficult to elucidate because of their unpredictable nature, but several hypotheses have been proposed to explain the mechanistic role of drug CRM in these cases, including the “hapten” and “danger” hypothesis (4, 7). The hapten hypothesis proposes the formation of protein adducts by irreversible, covalent binding of small molecules such as drug CRM to these proteins. Consequently these modified proteins will stimulate an immune response, as they are recognized as foreign to the patient’s body. However, many foreign proteins do not initiate an immune-mediated response without additional co-stimulatory signals activating the antigen-presenting cells. These co-stimulatory or “danger” signals may result from e.g. infection or disease, but could possibly also arise from cellular damage (e.g. oxidative stress) induced by the CRM.

It is clear that ADRs can have a large impact on both the output of the pharmaceutical research and development process and patients health. IDRs in particular are usually only discovered when the drug is already on the market, leading to costly drug withdrawal or regulatory black box warnings markedly restricting their usage. Attention thus focuses on limiting possible drug-related risk factors. As CRM are believed to play a key role in IDRs, early identification of such metabolites and study of their interaction with biological processes seems crucial. Patient-related risk factors rather than drug-related risk factors most likely tip the balance between bioactivation and inactivation of CRMs resulting in especially IDRs (8). The influence of such patient-related risk factors on
drug metabolism should therefore be carefully examined, e.g. by identification of the enzymes involved in the metabolism of certain drugs and their possible genetic polymorphisms resulting in interindividual differences in expression and/or activity levels of these enzymes. This chapter will briefly introduce drug metabolism in general, the available cellular models to study the biological consequences of drug metabolism, and some examples of the study of the cellular interplay between bioactivation, detoxification and transport in drug toxicity.

Figure 1. Role of CRMs in ADRs. Drug toxicity may occur through the accumulation of the parent drug or, via metabolic activation, through the formation of a CRM. If not detoxified, CRMs may covalently modify biological macromolecules resulting in cellular toxicity. Figure adapted from (9).

FORMATION & FATE OF CHEMICALLY REACTIVE DRUG METABOLITES

Drug biotransformation (phase I and phase II)

Enzymatic biotransformation (or: metabolism) of xenobiotic compounds, including drugs, plays an important role in protecting the human body from possible toxic effects of these foreign molecules. In general xenobiotic metabolizing enzymes catalyze the formation of metabolites with increased hydrophilicity compared to the respective parent compound, thereby resulting in increased excretion from the body (10). These enzymes can be grouped into two major types of reaction, namely modification of the parent compound by introduction of a new (or modified) functional group (commonly assigned “phase I”) or by conjugation with an endogenous polar moiety (“phase II” reactions such as glucuronidation, sulfation and glutathione conjugation).
Members of the cytochrome P450 (P450) family are primary contributors to the total phase I metabolism of drugs, responsible for about three quarters of the total metabolism of marketed drugs as illustrated in figure 2 (12). A range of minor contributing enzymes are responsible for the remaining phase I metabolism of drugs, including several dehydrogenases, esterases and oxidases, flavin-containing monooxygenase (FMOs) and NADPH:quinone oxidoreductase (NQO1) (11, 12). Phase II metabolism of drugs is dominated by UDP glucuronosyl transferases (UGTs), complemented by N-acetyltransferases (NATs), sulfotransferases (SULTs) and glutathione S-transferases (GSTs) (13). While only two NAT isoforms are known in humans, SULTs, UGTs and GSTs have a large number of (polymorphic) forms (13).

Figure 2. Major drug-metabolizing enzymes in the human body. ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; CYP, cytochrome P450; DPD, dihydropyrimidine dehydrogenase; NQO1, NADPH:quinone oxidoreductase or DT diaphorase; COMT, catechol O-methyltransferase; GST, glutathione S-transferase; HMT, histamine methyltransferase; NAT, N-acetyltransferase; STs, sulfotransferases; TPMT, thiopurine methyltransferase; UGTs, uridine 5′-triphosphate glucuronosyltransferases. Figure adapted from (11).

Although this broad range of biotransformation reactions will for the majority of xenobiotics result in the increased elimination of the drugs, they can in some cases activate the drug into a CRM. P450 enzymes are often key players in these bioactivation reactions. Probably the most-studied example in this regard is the P450-mediated activation of paracetamol (acetaminophen, APAP) into N-acetyl-p-benzoquinoneimine (NAPQI), the latter responsible for APAP-induced hepatotoxicity (14). Besides P450s other phase I and II metabolic enzymes have been implicated in the bioactivation of drugs, both by design and related to ADRs. For example the therapeutic activity of quinone-derived antitumor agents depends on the reduction (i.e. bioactivation) by the phase I enzyme NQO1; the resulting reactive hydroquinone metabolites will covalently bind to the host DNA (15). Glucuronidation by UGTs, usually considered an inactivation pathway, may lead to bioactivation of the often-prescribed non-steroidal anti-inflammatory drug diclofenac (DF) which results in reactive
acyl glucuronides that are able to covalently modify proteins, and are thereby believed to contribute to DF associated idiosyncratic hepatotoxicity (16, 17). Research in this thesis will focus on three classes of drug metabolizing enzymes which will be reviewed in this chapter in more detail: P450s, NQO1 and GSTs.

Cytochrome P450

The P450 enzymes catalyze a diverse range of reactions that may convert drugs into CRM. The majority of these reactions are oxidations, but a range of other reaction types has been reported (18). The human genome contains 57 functional P450 genes. Of these about a dozen isoforms belonging to the CYP1, CYP2 and CYP3 family are considered to be responsible for the biotransformation of xenobiotics (19). More specifically five members are responsible for about 95% of all P450-catalyzed drug oxidations: CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 (12). At clinically relevant concentrations many drugs tend to be metabolized by one or only a few P450 isoforms, stressing the importance of possible altered P450 activity and expression within the human population. A striking example is the CYP2C19 depended bioactivation of the antiplatelet agent clopidogrel. Defective genetic variations of the CYP2C19 gene (CYP2C19*2 and CYP2C19*3) fail to convert the clopidogrel prodrug into the active metabolite resulting in reduced effectiveness and increased risk of thrombosis (20). As a result the FDA issued a boxed warning to alert patients to the risk of reduced effectiveness and suggested genotyping of potential patients.

Almost all drug-metabolizing P450 enzymes are genetically polymorphic, and the allelic variation can be translated into four general phenotypes. Poor metabolizers carry two defective alleles and lack enzyme activity altogether, whereas intermediate metabolizers will either be heterozygous for the defective allele or can carry two allele variants that translate into a protein with lowered activity. Extensive metabolizers carry both functional alleles. Increasing the copy number of the functional gene will lead to ultra-rapid metabolism. CYP2C9, CYP2C19 and CYP2D6 are considered major players in this regard, as they are not only highly polymorphic but together also account for nearly 80% of total P450-mediated drug metabolism. However genetic polymorphisms of other CYP isoforms have also been described to influence clinical outcome of drugs. Bioactivation of the anticancer prodrug cyclophosphamide (CPA), for example, is highly variable in patients and has been attributed to mainly CYP2B6 (19). Therapeutic response appears to depend on the CYP2B6 genotype, e.g. a significantly increased rate of relapse in patients carrying the CYP2B6*5 variant. Extensive reviews of known clinical relevance can be found elsewhere (20–22). However, the interindividual differences in P450 levels and activity are considered to be multifactorial and are controlled by a broad range of factors including not only genetic polymorphisms but also sex, age and disease as is depicted in figure 3.
Figure 3. Factors contributing to interindividual variability in the P450-dependent biotransformation of drugs. A total of 248 known P450-dependent drug metabolism pathways were analyzed. The possibly increased and/or decreased activity resulting from these variability factors is indicated by arrows. Important factors are printed in bold. Adapted from (19).

NADPH:quinone oxidoreductase

Human NAD(P)H:quinone oxidoreductase 1 (NQO1) is a flavoenzyme capable of reducing a broad range of substrates via two-electron reduction. Known substrates include quinones, quinoneimines, glutathionyl-substituted naphthoquinones, azo- and nitro-compounds (23). Well-studied bioactivation reactions catalyzed by human NQO1 are those of antitumor drugs containing a quinone pharmacophore (figure 4A), such as mitomycin C, β-lapachone and 17-N-allylamino-17-demethoxygeldanamycin (17-AAG). Upon reduction by NQO1, some of these drugs form unstable hydroquinones that may either alkylate biomolecules like DNA, or undergo redox-cycling reactions resulting in the generation of reactive oxygen species (ROS) (15). In contrast, formation of a stable hydroquinone product by NQO1 may prevent toxicity of chemically reactive substrates like quinoneimine metabolites, or avoid one-electron reactions that may otherwise lead to reactive semiquinones and ROS (23). For example NAPQI, the reactive quinoneimine metabolite of APAP, can be reverted to APAP by NQO1 as is depicted in figure 4B (24). NQO1 is also involved in multiple cellular processes such as direct scavenging of superoxide anion radicals and the regulation of proteasomal degradation of specific proteins like the tumor suppressor p53 (25, 26).

NQO1 expression is regulated by the antioxidant response element (ARE) and can be strongly up-regulated by Nrf2/Keap1-mediated stress responses (23, 26). Two single nucleotide polymorphisms of NQO1 have been identified within the human population, C609T (NQO1*2) and C465T (NQO1*3).
The frequency of the NQO1*2 allele ranges from 16% in Caucasians to 49% in Chinese populations (27). Individuals carrying the NQO1*2/*2 genotype show very low to undetectable levels of the mutant protein, resulting from an accelerated degradation of the protein by polyubiquitination and subsequent proteasomal degradation (15). Heterozygous NQO1*1/*2 are believed to have a lower level of NQO1 expression compared to NQO1*1/*1 (28). The NQO1*2 polymorphism has been associated with an increased risk to benzene toxicity and increased susceptibility to cancer (29, 30). The effect of the NQO1*3 polymorphism on the phenotype is not extensively studied. Although comparable in stability to the wild type, changes in activity of the NQO1*3 mutant were shown, most notably a decreased activity for mitomycin C (31). The allele frequency of NQO1*3 is however much lower compared to the *1 and *2 alleles (32).

Figure 4. Role of NQO1 in bioactivation of bioreductive antitumor quinones (A), or inactivation of reactive drug metabolites like NAPQI (B). 17-AAG: 17-N-allylamino-17-demethoxygeldanamycin. Panel A adapted from (15).
Glutathione S-transferase

Glutathione S-transferases (GSTs) catalyze the conjugation of electrophilic xenobiotics with the endogenous tripeptide glutathione (GSH), thereby rendering the electrophilic compound inactive and facilitating its excretion (figure 5A). Human GSTs are divided in two superfamilies. The first group, the soluble GSTs, are dimeric enzymes subdivided into eight classes named alpha (A), kappa (K), mu (M), pi (P), sigma (S), theta (T), zeta (Z) and omega (O) (33). They are localized mainly in the cytosol, while GSTK1 has also been identified in mitochondria and peroxisomes (34). The other superfamily is formed by the “membrane-associated proteins in eicosanoid and glutathione metabolism” (MAPEG), also referred to as microosomal GSTs.

Figure 5. Different roles of GST pi (GST P1-1) in cellular response to electrophilic xenobiotics (such as CRM). GST pi catalyzes conjugation of xenobiotics to glutathione (A). Alternatively GST pi may protect proteins from the effects of oxidative stress by glutathionylation of cysteine residues (B). Moreover GST pi has been described to control the JNK/C-jun pathway (C). GST pi sequesters JNK/C-jun, thereby inhibiting JNK phosphorylation and preventing the activation of downstream kinases and transcription factors. Exposure to certain drugs or oxidative stress alters cellular redox homeostasis resulting in the oligomerization of GST pi and the dissociation of the complex, liberating JNK for phosphorylation. Panel B and C adapted from (35, 36).

Besides its catalytic function, GSTP1-1 has more recently been discovered to directly influence signalling pathways involved in cell survival (37). In addition GSTP1-1 was shown to significantly enhance S-glutathionylation of proteins in response to reactive oxygen and nitrogen species, both in vitro as well as in vivo (38). This post-translational modification of reactive cysteine residues in proteins, as depicted in figure 5B, has been proposed to play an important role in cellular redox homeostasis and the modulation of protein function. Bearing in mind the generally up-regulated expression of GTSP1-1 in cancer cells, it is interesting to note that many S-glutathionylated proteins are kinases involved in growth regulating pathways (39). The dual nature of GSTP is also illustrated by its role in hepatotoxicity upon APAP overdose. While GSTP1-1 is known to catalyze the GSH conjugation of NAPQI, P1-1 gene knockout in mice did not aggravate but rather protected from APAP-induced toxicity (40). Although the exact underlying mechanism remains to be elucidated,
the inhibitory role of GSTP in the Jun-terminal kinase (JNK) pathway (as illustrated in figure 5C) was suggested to potentiate APAP toxicity.

Most of the cytosolic GST isoforms are known to be polymorphic within the human population and may affect disease- and drug susceptibility (33, 41). Common gene deletions of GSTM1 (present in about 50% of both Caucasian and Asian population) and GSTT1 (ranging from a frequency of 20% in Caucasians to 50% in Asian populations) will result in a complete loss of enzyme activity which is e.g. associated with a small increased risk of certain cancers (42). These GSTM1 and GSTT1 null genotypes have also shown significant association with hepatotoxicity induced by several drugs, including carbamazepine, tacrine and troglitazone (43–46). In contrast, the four allelic variants of GSTP1-1 have been shown to have a different pharmacokinetic profiles towards several CRMs, but this catalytic effect is relatively minor and unlikely to be a major factor in determining clinical outcome (47).

Active transport (phase 0 and phase III)

The classical division of drug metabolism in two phases has been refined in later years to include the fact that active transport of xenobiotics contributes to the clearance from the body by regulating cellular uptake and efflux of drugs and drug metabolites (48). By now, these two additional steps in the metabolism of drugs are called “phase 0” and “phase III”. In phase 0 cellular entry and exit of the unmodified drug is modulated, while phase III describes the efficient elimination of already metabolized drugs from the intracellular compartment. Transporter proteins involved in these reactions are often divided in two major groups. The first group is comprised of the solute carrier (SLC) transporters that facilitate the transport of a wide range of substrates without the use of ATP. They include several well-characterized organic anion transporter proteins (OATPs or SLCOs) known to be polymorphic and involved in the pharmacokinetics of a range of known drugs, most notably a wide range of statins (49,50). The relationship between genetic OATP polymorphisms and a clinical toxicological outcome is nicely illustrated by the case of simvastatin-induced myopathy, which was shown to be strongly associated with an OATP1B1 single nucleotide polymorphism (49, 51).

A second group is formed by the ATP binding cassette (ABC) proteins. ABC transporters are typically known as membrane proteins involved in the acquired resistance of patients towards multiple anticancer drugs in cancer treatment (52). These transporters are therefore historically known as multidrug resistance (MDR) proteins. In humans the major MDR-ABC proteins include MDR1/P-glycoprotein (ABCB1), several members of the MRP (ABCC) subfamily and the MXR/BCRP (ABCG2) transporter (48). Their wide range of substrates generally consists of hydrophobic compounds, although MRP1 (ABCC1) and ABCG2 are also able to transport amphiphatic charged compounds such as glutathione- or glucuronide-conjugates of drugs (53,54). ABC transporters also show genetic variation within the human population (50), but their true clinical impact is still under debate (55).
Interplay between drug metabolizing enzymes and transporter proteins, either directly through their catalytic function or indirectly via cellular signalling, will determine the formation and fate of CRMs. However, the work in this thesis focuses primarily on the catalytic interplay between multiple drug metabolizing enzymes (specifically CYPs, GSTs and NQO1) and the subsequent effects on cell viability.

**IN VITRO CELLULAR MODEL SYSTEMS TO STUDY EFFECTS OF DRUG BIOACTIVATION**

The early identification of reactive drug metabolites and the study of their interaction with biological processes could prevent ADRs in the later stages of drug development. Additionally, knowledge of the different metabolic pathways involved in ADRs or IDRs of specific drugs could aid a pharmacogenetic-guided approach where patients are genetically screened for possible polymorphisms of the drug metabolizing enzymes involved, making personalized pharmacotherapy possible \(^{(22)}\). However, such studies to elucidate metabolism-related toxicity are complex. Animal models used in preclinical toxicity studies have limited predictability for clinical ADRs and IDRs in particular, and seems to vary significantly between the type of toxicity \(^{(56–58)}\). Interspecies differences in the expression levels, catalytic activity and tissue distribution of drug metabolizing enzymes and drug transporters are likely to be the underlying cause of this problem \(^{(59)}\). More recent advances in animal models engineered to mimic human disease have meanwhile shown to be better predictors of some IDRs \(^{(60)}\). However, *in vivo* animal studies are costly in both time and financial expenses and are under ethical debate.

Many cellular *in vitro* model systems have been developed to elucidate pathways of drug-metabolism, but these *in vitro* results are not easily translated to the *in vivo* situation because the latter tends to depend on patient-related risk factors causing interindividual variation of enzyme activities controlling the biological fate of the drug and its metabolites, including many genetic polymorphisms \(^{(8, 20, 22)}\). Still, they may contribute to the three R’s in animal testing (reduction, replacement and refinement) and help to overcome limitations like interspecies differences in toxicity testing. Different types of cellular model systems differ in their biological complexity, ease of use, ethical acceptability and resemblance of the actual human *in vivo* situation \(^{(61)}\). These *in vitro* models are usually a compromise between convenience and relevance, as is depicted for cellular models for liver toxicity in figure 6. Biologically more complex cellular systems resemble the human body more closely, but these are generally more difficult to handle and more expensive due to limited availability. Finally, only complex, multicellular models including immune cells might be suitable to successfully predict the HLA-restricted phenotype of certain ADRs in the future.
Cellular model systems expressing endogenous biotransformation enzymes

Because the liver is generally considered the primary site of drug metabolism, *in vitro* systems for drug-metabolism studies should preferably be derived from human-liver samples (62, 63). Currently, primary human hepatocytes are considered the golden standard as they contain all drug metabolizing enzymes and necessary cofactors (64, 65). But, to confirm metabolism-dependent toxicity, parallel studies have to be performed using metabolically incompetent cells or by using chemical inhibitors. The latter may however complicate results, as chemical inhibitors may also affect other cellular processes and thus drug toxicity. For example, several P450 inhibitors are known to influence drug transport and glucuronidation (66, 67). In addition, P450 expression in hepatocytes declines both in suspension (which is preceded by a rapid initial loss of mRNA of up to 40% after 4 to 6 hours) and in attached monolayer cultures (approximately 50% per day), and this decrease is shown to be different for individual isoenzymes (65, 68). Differences in P450 expression between donors is fairly extensive, making *in vitro* - *in vivo* extrapolation even more difficult (69). Cryopreservation of hepatocytes aids their availability and ease of use. Although phase II metabolic enzyme activities (including UGT, SULT and GST activity) are well maintained, intracellular glutathione levels are however drastically reduced (68). More recent advances include 3D culturing to better mimic *in vivo* structure and function of the human liver, and microfluidic systems that allow the 3D cellular environment to be modelled on a microchip (70). Besides primary hepatocytes, precision cut liver slices (PCLS) also retain phase I and phase II metabolism (71, 72). To date, the decrease of metabolic capacity over time is still somewhat ambiguous (70). PCLS do however offer an additional advantage over cultured hepatocytes because cell-cell connections are preserved (62).

Immortalized cell lines derived from human liver are available unlimited and easy to use, but the expression of many drug metabolizing enzymes is frequently poorly characterized and if characterized often shown to be very low (73). This is especially true for the expression of P450 enzymes in well-known cell lines such as HepG2, while the expression of phase II enzymes is much more comparable to primary human hepatocytes (74, 75). The more recent HepaRG cell line does show improved stable expression of P450 enzymes, though expression levels are still lower for most isoforms compared to isolated hepatocytes (76–78).
Chapter 1
Extracellular bioactivation of drugs using sub-cellular preparations

Extracellular or exogenous bioactivation systems can be used to overcome insufficient activity of drug-metabolizing enzymes in cell lines. Extracellular bioactivation of drugs can be performed by specific isolated drug metabolizing enzymes, either prepared from biological tissues (e.g. rat or human hepatic S9 fraction and liver microsomes) or by heterologous expression and isolation of (human) enzymes (recombinant enzymes). A well-known example of exogenous bioactivation is the use of rat hepatic S9 fraction in many genotoxicity studies where metabolic activation of tested compounds is required (79). However the human relevance of this metabolic system needs to be questioned. P450 enzymes present in rat liver S9 fraction differ in substrate selectivity and activity (in particular the CYP2 family), and although the rat S9 mix includes phase II metabolic enzymes, the required cofactors are usually not added upon incubation (80). In a similar fashion, cell lines can be co-incubated with isolated recombinant expressed human enzymes, which are potentially much cleaner compared to rat liver S9 and can be used to investigate a very specific metabolic pathway. Indeed co-incubation of HepG2 cells with CYP3A4 supersomes (microsomes derived from baculovirus-infected insect cells that show very little endogenous phase I and phase II metabolism) produced amiodarone- and clopidogrel-induced cytotoxicity that was comparable to HepG2 cells expressing intracellular CYP3A4 (81, 82). Bioactivation of the anticancer prodrug cyclophosphamide (CPA) and APAP by CYP3A4 and CYP2B6 supersomes resulted in clear cytotoxicity in MCF7 breast cancer cells cultured on a microchip (83). Similar microfluidic devices have successfully been used in combination with human liver microsomes (HLM) to induce APAP-toxicity in HepG2 cells (84, 85). However, extracellular bioactivation does pose a potential problem for drug metabolites that are too reactive or hydrophilic to enter the cell; in these cases intracellular biotransformation needs to be enhanced instead.

Genetically engineered cell lines to study toxicity of drug metabolites

In contrast to exogenous bioactivation methods, genetically engineered cells that express drug metabolizing enzymes ensure the generation of drug metabolites in close proximity to the target site of toxicity (73). Especially for CRM, this method of intracellular bioactivation is thus more likely to lead to toxicity. Comparison of extracellular bioactivation of CYP3A4 supersomes co-incubated with HepG2 cells and intracellular bioactivation by HepG2 cells transfected with CYP3A4 yielded comparable cytotoxic results for a panel of 11 drugs (86). Carbamazepine, however, was only able to induce cytotoxicity in the CYP3A4 transfected HepG2 cells, most likely because it is metabolized into several unstable and highly reactive epoxide and iminoquinone metabolites, that are unable to reach the intracellular target after exogenous bioactivation (86). A variety of mammalian cell lines have successfully been used to express all major human P450 genes, either alone as specific P450 isoforms and in combination (87). Such cell lines can be used to identify the pathways involved in the metabolism of specific drugs and create metabolic profiles. Examples include the identification of isoform-specific contribution of P450 enzymes in the formation of hydroxylated metabolites of DF (88) and the determination of intrinsic clearances of four selected P450 substrates (phenacetin,
tolbutamide, alprazolam and midazolam) (89). Moreover, mammalian cell lines engineered to express bioactivating enzymes can be used to assess cellular responses to (reactive) drug metabolites, such as drug-induced genotoxicity or genotoxicity. Benzo[a]pyrene and CPA were able to increase micronucleus induction (cytoplasmic bodies containing chromosomal fragments or whole chromosomes) in HepG2 cells expressing particular P450 enzymes involved in the bioactivation of these compounds (90). Other bioactivating enzymes have also been expressed in mammalian cell lines. For example, Glatt et al. developed a Chinese hamster V79-derived cell line co-expressing CYP2E1 and SULT1A1, two human enzymes involved in the bioactivation of numerous promutagens and procarcinogens (91). Using this model, they were able to show greatly enhanced genotoxicity of several food-borne chemicals, including N-nitrosodimethylamine, 5-hydroxymethylfurfural and the herbicide nitrofen. However, these chemicals are bioactivated by either CYP2E1 or SULT1A1 pathways, and their genotoxicity does thus not rely on the combination of both metabolic enzymes. In addition, HepG2 cells transfected with individual P450 isoforms confirmed the ability of aflatoxin B1, APAP, CPA and DF to reduce cell viability upon their bioactivation (92).

A more refined cell-based screening assay for cytotoxicity in HepG2 cells has been developed and tested for a large set of 66 hepatotoxic compounds (93). This high content screening (HCS) assay uses a combination of five parameters: not only cell viability, but also nuclear morphological changes, lipid peroxidation or reactive oxygen species (ROS) formation, alterations of the mitochondrial membrane potential, and intracellular calcium concentration. Simultaneous expression of five P450 enzymes within this cellular HCS model significantly decreased IC$_{50}$ values for twelve drugs with known bioactivation-dependent toxicity. Moreover, observed changes in the analyzed parameters for these drugs were in good agreement with their previously reported mechanisms of toxicity (94). Integrated safety assessment of drug CRM may be achieved by joining multiple cell-based assays into a in vitro test panel for drug safety (6). One such integrated approach was proposed by 1. comparing a metabolically inactive THLE cell line with THLE cell lines expressing CYP3A4, 2. cytotoxicity screening in HepG2 cells cultured in both glucose and galactose media (indicative for mitochondrial injury), 3. inhibition of human bile salt exchange pump and rat Mrp2, and 4. covalent binding of radiolabeled compound to human hepatocytes (3). This in vitro panel was successful in discriminating drugs with a high IDR risk from those with low IDR concerns.

The Nrf2 regulatory pathway senses chemical and oxidative stress and consequently induces several protective enzymes including heme oxygenase-1 (HO-1), NQO1, GSTs, SULTs, UGTs and MRPs (95). Such oxidative stress factors include GSH depletion and ROS formation, toxicological events that may be associated with CRMs (10, 96, 97), while CRMs themselves may directly bind to the Keap1 repressor protein. Figure 7 shows a simplified scheme of APAP metabolism and the involvement of the Nrf2-Keap1 pathway. The Nrf2 transcription factor might thus be another potential indicator of toxicity in cell lines, but its predictive remains to be validated (98). Incubation of HepaRG cells with the anti-psychotic drug clozapine (CLZ) showed a marked increase in HMOX1 mRNA expression indicating that the HepaRG cell displays a functional Nrf2 pathway and may possibly be used as a predictive tool (98). Induction of HO-1 was also observed for IDR-related drugs (including DF and CLZ) in human hepatocytes (99). To facilitate the measurement of cellular
Nrf2 responses reporter-constructs have been constructed, such as the fluorescent ToxTracker and chemiluminescent CALUX assay \((79, 100)\).

Although mammalian cell lines are a valuable tool in the safety assessment of drugs, they grow slowly and their genetic modification is more difficult compared to microbial cells. Moreover, the choice of mammalian cell type can significantly influence the cellular response towards toxic compounds. For example, frequently used p53-deficient rodent cell lines (including V79 and CHO cells) are more sensitive towards cytotoxicity and genotoxicity compared to p53-competent cells like HepG2 \((101)\). As several signalling pathways are known to exhibit cross-talk, like interactions between the p53- and Nrf2-pathway \((102)\) as indicated in figure 7, such genetic variation of signalling proteins possibly influences cell defence mechanisms on a broader scale.

![Figure 7](image)

**Figure 7.** Simplified scheme illustrating the role of the Nrf2 transcription factor in APAP metabolism. APAP primarily undergoes sulfation and glucuronidation, though at high dose CYP metabolism into the reactive NAPQI metabolite becomes more prominent. NAPQI can subsequently be reduced to APAP by NQO1, be detoxified by GSH conjugation which possibly results in GSH depletion, or covalently modify proteins such as Keap1. The latter two events stimulate the release of Nrf2, which translocates into the nucleus and initiated transcription of a wide range of cellular defence genes under the control of the antioxidant response element (ARE), including the detoxifying enzymes and transporters involved in APAP metabolism. Cross-talk between the Nrf2- and p53-pathway may coordinate the toxicological outcome: cell survival or cell death \((103)\).

**In silico prediction of human exposure: in vitro - in vivo extrapolation**

As the drug metabolizing potential of the cellular models described above cannot accurately reflect the drug metabolism variability in the human population, data from *in vitro* experiments needs to be extrapolated. Combined *in silico* and *in vitro* methods are increasingly being recognized as
potential tools to assess drug safety without animal testing (63). This has led to the development of many physiologically based toxicokinetic (PBTK) models, in which drug metabolism has been incorporated to ensure a more complete picture (64, 104). These mathematical models usually aim to describe exposure of specific target tissues to drugs and/or their metabolites. Once *in vitro* effect concentrations have been established, quantitative extrapolation will yield *in vivo* toxic dose levels. More recent advanced models (as illustrated in figure 8) also include phase 0 and phase III ABC transporters and a (rather basic) transcriptional-translational feedback loop in the form of the Keap1-Nrf2 pathway controlling cellular oxidative stress response (105). Some examples of PBPK modelling include the study of APAP-induced hepatotoxicity (106, 107).

In general, building a PBTK model requires both data on the system (e.g. physiological and biochemical data), drug-specific data and the model structure (e.g. the arrangement of tissues and organs) (108). *In vitro* kinetic data, such as $K_M$ and $V_{max}$ values, on drug metabolism can be determined in cellular models, but also using extracellular methods using isolated enzymes or liver fractions. However scaling factors (or intersystem extrapolation factors, ISEFs) are needed to translate protein expression and activities (109).

The work described in this thesis assesses cellular toxicity of CRMs following both extracellular bioactivation (by isolated CYPs added to the culture medium) and intracellular bioactivation (by heterologous expression of CYPs in a cellular yeast model). In addition kinetic parameters of APAP metabolism by human CYPs and GSTs were determined to facilitate *in silico* modelling of cellular exposure to the reactive NAPQI metabolite within the Dutch Assuring Safety without Animal Testing (ASAT) program.
CURRENT MAMMALIAN CELLULAR MODELS TO STUDY METABOLISM-RELATED INTERPLAY IN DRUG TOXICITY

The different metabolic pathways of a drug will influence the interplay between CRM formation, inactivation and transport. This balance between the four phases in drug biotransformation and disposition may therefore ultimately dictate the exposure of the body to the reactive metabolite and thereby affect the toxicological outcome. Although many different in vitro models are available to study the biotransformation of drugs within a cellular context, examples that focus their attention to a direct effect on drug toxicity caused by interplay in drug metabolism are still rather scarce. This section will briefly describe a selection of examples from literature, summarized in table 1.

Table 1: overview of cellular models studying metabolism-related interplay in drug toxicity.

<table>
<thead>
<tr>
<th>Cellular model (interplay studied)</th>
<th>Compounds</th>
<th>Methods</th>
<th>Results</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>HepG2 (CYP/UGT/GST)</td>
<td>tamoxifen safrole</td>
<td>HepG2 transformants expressing various CYPs (2A6, 2C9, 2D6, 2E1, 3A4) were treated with DF to inhibit endogenous UGTs or ethacrynic acid to inhibit endogenous GSTs.</td>
<td>Micronucleus induction due to CYP bioactivation increased after inhibition of UGT- and GST-pathways</td>
<td>(110)</td>
</tr>
<tr>
<td>HepG2 (CYP3A4/Nrf2)</td>
<td>selection of 23 hepatotoxic drugs</td>
<td>HepG2 infected with 3A4 adenovirus; subsequent transfection with siNrf2.</td>
<td>Several drugs (CLZ, flutamide, troglitazone) only showed CYP-dependent cytotoxicity upon inhibition of Nrf2-controlled defence genes.</td>
<td>(111)</td>
</tr>
<tr>
<td>V79 (CYP1A1/GSTP1-1)</td>
<td>benzo[a]pyrene dibenzo[a,l]pyrene</td>
<td>V79 transfected with CYP1A1 alone and combined with GSTP1-1.</td>
<td>Cytotoxicity induced by expression of CYP1A1, subsequent protection by co-expression of GSTP1-1</td>
<td>(112)</td>
</tr>
<tr>
<td>MCF7 (MRP1/GSTA1-1)</td>
<td>chlorambucil</td>
<td>MCF7 expressing MRP1 and GSTA1-1 alone and in combination</td>
<td>Only full resistance to chlorambucil upon combined expression of MRP1 and GSTA1-1.</td>
<td>(113)</td>
</tr>
<tr>
<td>Caco-2 (CYP3A4/MDR1)</td>
<td>K77 sirolimus</td>
<td>CYP3A4 transfected Caco-2 cells; selective chemical inhibition of MDR1.</td>
<td>Inhibition of MDR1 increased intracellular levels of parent compounds and CYP-catalyzed metabolites</td>
<td>(114–116)</td>
</tr>
<tr>
<td>HEK293 (OATP1B1/ CYP3A4/MDR1)</td>
<td>atorvastatin acid atorvastatin lactone</td>
<td>HEK293 stably transfected with OATP1B1 co-transfected with CYP3A4 and MDR1 in different combinations.</td>
<td>Intracellular levels of CYP-catalyzed atorvastatin acid metabolites dependent on active uptake by OATP1B1 and efflux by MDR1. Levels of more lipophilic atorvastatin lactone metabolites not influenced by active transport.</td>
<td>(117)</td>
</tr>
<tr>
<td>HEK293 (NQO1/UGT1A6/ UGT1A10)</td>
<td>menadione</td>
<td>Expression of NQO1 alone and in combination with UGTs.</td>
<td>NQO1-mediated activation of menadione results in cytotoxicity; expression of UGTs mitigated cytotoxicity by glucuronidation of the menadiol metabolite. Release of GSH-conjugates of menadione detected, indicating possible role of GSTs.</td>
<td>(118)</td>
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</table>
P450 enzymes often play a pivotal role in the formation of drug CRM leading to toxicity, but heterologous expression of P450 enzymes in immortalized cell lines is often required for sufficient bioactivation of drugs into CRMs due to their low intrinsic activity in immortalized cell lines. However, to study potential interplay of CYP-mediated bioactivation and phase II metabolism overexpression of phase II enzymes is not always required. Hashizume et al. were able to show an interaction between phase I and phase II metabolism in HepG2 transformants expressing major human P450 isoforms using tamoxifen and safrole. The antiestrogen tamoxifen requires bioactivation through CYP3A4 hydroxylation and subsequent SULT-mediated conjugation to form the ultimately genotoxic sulfate ester, though the intermediate hydroxylated tamoxifen metabolite can be detoxified by UGT-catalyzed glucuronidation. A similar cascade of P450 hydroxylation (by predominantly CYP2A6, 2C9, 2D6 and 2E1) and SULT-mediated conjugation converts the compound safrole into a genotoxin, but in parallel a detoxifying role of GST-catalyzed GSH conjugation is reported. Hashizume observed micronucleus induction after inhibition of the detoxifying phase II pathways. When DF was co-administered with tamoxifen as an UGT-inhibitor, or when GST-catalyzed conjugation of safrole metabolites was inhibited by ethacrynic acid, genotoxicity was observed.

Hosomi et al. tested 23 drugs known to cause hepatotoxicity in HepG2 cells infected with a CYP3A4 adenovirus. Many of these drugs significantly reduced cell viability, but surprisingly a number of drugs failed to show an increase in cytotoxicity by CYP3A4 expression, even though the underlying mechanism of their toxicity is known to be dependent on CYP3A4-metabolism (e.g. CLZ, flutamide and troglitazone). Transfection of these HepG2-CYP3A4 transformed cells with siNrf2, decreasing the expression of the Nrf2 transcription factor regulating many antioxidant proteins and phase II enzymes, resulted in an exacerbated CYP3A4-mediated cytotoxicity which suggests an important role of NQO1- and phase II metabolism in the cellular sensitivity towards CYP-generated CRMs.

GSTs have long been associated with drug resistance in cancer chemotherapy and have gained much attention. P450- and GST-related interplay e.g. involves the balance between the bioactivation and bioinactivation of polycyclic aromatic hydrocarbons. By transfecting V79 cells with human CYP1A1 a clear P450-mediated cytotoxicity was induced by benzo[a]pyrene and dibenzo[a,l]pyrene. Co-expression of human GSTP1-1 resulted in a convincing resistance towards the P450-dependent drug toxicity. But overexpression of GSTs does not necessarily confer resistance to anticancer drugs. Transporter-mediated efflux of glutathione-conjugates of these reactive drugs has been proven to be necessary to fully potentiate GST-mediated protection in some cases. Using MCF7 breast carcinoma cells expressing MRP1 (ABCC1) and GSTA1-1, both separately and in combination, it was shown that both proteins needed to be active to confer full resistance against chlorambucil, illustrating an interesting synergy between phase II and phase III pathways in drug toxicity. The role of such combined action (and regulation) of phase II enzymes and transporters of the MRP (ABCC) family in multidrug resistance in cancer has been reviewed elsewhere.
As CYP3A and MDR1 (P-glycoprotein, ABCB1) substrates show considerable overlap, their interplay has been explored in detail during the last two decades (114–116, 120). CYP3A4-transfected Caco-2 cells were used in combination with CYP3A4 substrates (midazolam and felodipine) and combined CYP3A4/MDR1 substrates (K77 and sirolimus). Results suggested that selective inhibition of MDR1 would increase exposure of the dual substrates (K77 and sirolimus) to intestinal CYP3A4 (114, 115). Moreover, intracellular levels of sirolimus metabolites formed by CYP3A4-metabolism were increased, indicating that their removal was dependent on MDR1 activity (116). Likewise, intracellular levels of atorvastatin acid metabolites have recently been shown to be dependent on both OATP1B1 uptake, 3A4 metabolism and MDR1 efflux in a HEK293 model (117). In contrast, using this same cellular model, metabolism of the more lipophilic atorvastatin lactone was independent of active transport. Several more transporters have been described to interact with phase I and phase II metabolism of drugs (e.g. CYP3A/SLCOs and UGTs/ABCC2), but these interactions have generally been investigated in animal models or clinical studies (121).

Interplay between phase I enzymes and conjugating enzymes or transporter proteins is not limited to enzymes of the P450 family. NQO1 is known to activate menadione to menadiol, which can be reverted to the parent drug with simultaneous formation of ROS; this continuous redox cycling is believed to be responsible for cytotoxicity. Glucuronidation of menadiol subsequently prevents this redox cycling. Indeed NQO1-mediated activation of menadione was mitigated by glucuronidation of menadiol in a HEK293 cell line co-expressing NQO1 with UGT1A6 and UGT1A10 (118). Moreover, wild-type HEK293 cells in this study released glutathione-conjugated menadione, suggesting that GSTs may influence the balance between CRM formation and detoxification.

In this thesis interplay between bioactivation and inactivation of drugs is assessed both using isolated enzymes (by combining recombinant CYP and NQO1 enzymes) and in a cellular context (by expressing CYP and GST enzymes in yeast cells).

**YEAST AS A CELLULAR MODEL IN DRUG SAFETY STUDIES**

The yeast *Saccharomyces cerevisiae* (to which we specifically refer to as “yeast” in the remainder of this thesis) is a model eukaryote popular for its genetic accessibility, genome-wide screens, cost-effectiveness and rapid growth (122). We will briefly highlight the potential use of yeast to study biotransformation-related toxicity of drugs in comparison with mammalian cellular models described above. Several reviews of yeast as a cellular model system in drug discovery are available (122–125). One-third of known genes involved in human disease have functional yeast homologues, and hundreds of yeast genes are described to have a link to human disease genes (125, 126). Detailed screening for disease genes associated with human mitochondrial deficiencies showed not only high similarity to yeast mitochondria but also identified possible candidate genes that may be involved in man (127). Also, formation of ROS and the effects to the cell has been studied well in yeast, but also shows promising potential as a tool to study the molecular mechanisms underlying human pathologies related to oxidative stress (97, 128, 129).
Chapter 1
General introduction

The systematic construction of a collection of gene-deletion strains has greatly aided the use of yeast for on- and off-target identification (130). Using yeast as a model organism for pharmacogenetic studies, 214 psychoactive drugs were screened for possible off-target effects (131). Of these compounds, 81 showed growth inhibition in wild-type yeast and were selected for profiling using a collection of yeast deletion strains, resulting in the identification of cellular processes that interacted with the tested compounds, including several clinically relevant drugs such as CLZ and fluoxetine. CLZ was later discovered to interact with yeast COX17, a cytochrome c oxidase copper chaperone (132). Whereas deletion of the yeast COX17 gene resulted in sensitivity towards CLZ, subsequent expression of the human Cox17 protein conferred resistance. High conservation between mammalian and yeast mitochondrial disease genes (127) enabled the mechanistic study of mitochondrial toxicity of drugs like DF (133). Similar chemical-genetic screening revealed striking differential toxic effects of two structurally very similar compounds (an imidazo[1,2-a]pyridine and -pyrimidine); the pyridine acted on mitochondrial targets while the pyrimidine caused nuclear DNA damage (134).

Besides target identification, several cellular reporter systems for toxicity have been developed in yeast. The yeast estrogen screen (YES), a system expressing the human estrogen receptor combined with an estrogen response element reporter-gene, is an established method for detecting endocrine disrupting chemicals in e.g. wastewater that is still being refined (135–137). The GreenScreen assay is based upon a reporter-gene (green fluorescent protein, GFP) under the control of a RAD54-promoter that detects DNA damage and is suggested to be highly sensitive and specific towards carcinogens when combined with an Ames test (138–140). Moreover, a GreenScreen derived model has been developed in a human lymphoblastoid TK6 cell line combined with an S9 metabolic activation assay (141).

*S. cerevisiae* only has three endogenous P450 enzymes (142). These three enzymes are all involved in cellular housekeeping activities; their involvement in xenobiotic metabolism has not been described except for the biotransformation of benzo[a]pyrene by CYP61 (123, 143). Heterologous expression of human P450 enzymes in yeast is however common. Many of these genetically modified yeasts are employed as bioreactors for the synthesis of drug metabolites and specific biological compounds (144–147). For example, expression of CYP2C9 facilitated the gram-scale formation of 4'-hydroxydiclofenac metabolites (148). Although less common, P450-mediated toxicity of drugs in yeast has been described. An increase in mutation frequency was observed in yeast cells treated with CPA upon expression of rat CYP2B1 (149). Genotoxicity of aflatoxin B1 requires P450-bioactivation, and indeed only by introducing human CYP1A2 this genotoxicity was observed in yeast (150, 151). Also the GreenScreen assay has successfully been combined with human CYP3A4 and CYP1A2 expression to bioactivate CPA and aflatoxin B1 into their genotoxic metabolites (152). One of the rare examples of metabolism-related toxicity of non-genotoxic drugs in yeast is our study of DF. We have shown that DF-induced growth inhibition and ROS formation was increased by introducing P450-metabolism (153). For this a bacterial P450 BM3 mutant, engineered to metabolize several drugs into human relevant metabolites at high catalytic rate, was expressed (154). Similar increased toxicity of NSAIDs structurally related to DF was observed in this yeast P450 BM3 model (155).
S. cerevisiae does not express any endogenous proteins that can be classified as UGT or SULT homologues. Yeast does however express several endogenous enzymes classified as GSTs, of which some have been described to play a role in the protection against oxidative- and xenobiotic-induced stress (156, 157). Mammalian phase II enzymes have previously been introduced in yeast cells. Expression of hGSTA1-1 and hGSTP1-1 has been shown to protect against several antitumor drugs (158). Several mammalian UGTs and SULTs have been expressed (159–161). Co-expressions of phase I and phase II metabolic enzymes have also been performed in yeast, such as the CYP BM3 M11 mutant with human SULT1A1 (J.S. van Leeuwen, D.M. Vredenburg-Maasdijk, unpublished results), and rat CYP1A1 together with UGT1A6 (162), but their use in drug toxicity studies has not been reported. To our knowledge our co-expression of P450 BM3 and human GSTs and their effects on clozapine toxicity in yeast is the first example of its kind (163).

For reasons described above, the yeast S. cerevisiae was chosen as a fast and cost-effective cellular model system in this thesis, potentially suitable for high-throughput use and investigation of mechanisms underlying ADR.

AIMS AND OUTLINE OF THIS THESIS

Aims of the thesis

Drug metabolism leading to ADRs and IDRs is a major cause of drug failure during development and clinical use (1). Different metabolic pathways involved in drug metabolism, e.g. involving CRMs, will influence the interplay between CRM formation, inactivation, cellular signalling and transport. This balance between the four phases in drug disposition, illustrated in figure 9, will ultimately dictate the exposure of the body to the reactive metabolite and thereby affect the toxicological outcome. Correlation of the expression of individual enzymes with toxicity is an important tool to allow the explanation of extreme sensitivity of a small group of patients to a particular drug. This knowledge is a prerequisite for the development of personalized medicine and safety, including the prevention of ADRs and IDRs.

The general objective of this thesis was to study the interplay of phase I and phase II drug metabolizing enzymes and possible subsequent effects on drug toxicity. The work presented in this thesis can broadly be divided in two types. Firstly, we set out to genetically engineer the yeast S. cerevisiae as a cellular model system to study P450-mediated bioactivation of drugs into toxic CRMs, either alone or combined with subsequent protective GST-catalyzed detoxification, both intracellularly. Secondly, we used isolated enzymes to study drug bioactivation and inactivation pathways (e.g. by human NQO1 and GSTs) and their possible interplay outside of the cell, i.e. extracellularly.
Figure 9. Yeast model depicting possible interplay between metabolic enzymes in drug toxicity. In this picture the drug is activated by a P450 enzyme into a CRM within the cell. The CRM will consequently lead to toxicity, or is inactivated by phase II conjugation (e.g. GST catalyzed glutathione conjugation). In addition, transporter proteins may influence cellular exposure to the drug (thereby affecting availability of the drug as a substrate for bioactivation) and it’s metabolites.

Outline of the thesis

The current chapter, chapter 1, provides a general introduction into the work presented in this thesis. The role of reactive drug metabolites (CRMs) in ADRs and important metabolic pathways are briefly discussed. Several popular cellular models to study metabolism-related toxicity are reviewed, including examples that study interplay between multiple drug metabolizing enzymes and/or transporter proteins. Lastly, we will discuss the use of the yeast *S.cerevisiae* as a cellular model in drug safety studies.

Chapter 2 describes the P450-mediated toxicity of diclofenac (DF) in yeast genetically modified to express the highly active P450 BM3 mutant M11. The bacterial P450 BM3 (CYP102A1) from *Bacillus megaterium* is a soluble single polypeptide in which the catalytic heme-domain is fused to a mammalian-like di-flavin NADPH-CYP reductase. With the highest activity ever reported for a cytochrome P450 monooxygenase, it has frequently been studied as a possible biocatalyst for many non-natural substrates. Our lab previously engineered several mutants showing conversion of over 40 drugs and drugs-like compounds, some at very high catalytic rate and producing human-relevant metabolites (154, 164, 165). Intracellular formation of human-relevant oxidative DF-metabolites...
by the P450 BM3 M11 mutant inhibited growth and increased the formation of ROS. A role of mitochondrial dysfunction in DF-induced toxicity was observed.

In chapter 3 a P450-BM3 expressing yeast strain was expanded by co-expressing human GSTs. Clozapine (CLZ)-induced growth inhibition was increased by P450 BM3 M11. While human GSTA1-1 and GSTM1-1 did not show any protection towards CLZ-induced growth inhibition, GSTP1-1 did. Co-expression of selected human GSTs resulted in intracellular formation of isoform-specific CLZ-glutathione conjugates. Formation of ROS upon CLZ exposure was apparent in wild-type yeast, was increased by P450 BM3 metabolism, but was not mitigated by GSTP1-1.

New possible interactions between drug bioactivation and -inactivation was also studied outside of the cell using isolated enzymes. In chapter 4 we show that P450-formation of reactive quinoneimine metabolites of three model drugs (APAP, DF and mefenamic acid) was balanced by human NQO1-catalyzed reduction back to the parent drug. Moreover NQO1 was able to effectively compete with GSTP1-1 catalyzed glutathione conjugation of the involved CRMs. Kinetic parameters of APAP bioactivation by human CYPs and glutathione conjugation of the reactive NAPQI metabolite by human GSTs were determined as well.

The anticancer prodrug cyclophosphamide (CPA) has frequently been studied in light of drug-resistance in chemotherapy. Chapter 5 describes the screening of a bacterial P450 BM3 mutant library for the bioactivating potential of CPA and ifosfamide (IFA). Two highly active BM3 mutants were identified. The two mutants were subsequently used for exogenous bioactivation (an alternative to less defined methods such as liver S9 fractions), which resulted in clear CPA- and IFA-induced cytotoxicity in human U2OS cells. We suggest that the identified mutants might possibly be used within a P450-based gene-directed enzyme prodrug therapy (GDEPT) to improve local bioactivation of CPA and IFA, thereby limiting associated ADRs. Since a highly active bioactivation method for CPA and IFA is now readily available, this may aid the investigation of possible interplay with e.g. GST-catalyzed glutathione conjugation.

In chapter 6, we evaluated the use of yeast and bacterial Escherichia coli cells expressing P450 BM3 mutants for whole-cell biosynthesis of drug metabolites. Large-scale synthesis of such metabolites may aid their structural elucidation and serve as analytical standards or novel lead compounds during the drug discovery and development process. Using the progestin norethisterone as a model drug we showed that yeast could successfully be used for whole-cell biocatalysis of norethisterone metabolites, although E.coli was more convenient and efficient.

Finally, in chapter 7 we summarize and discuss the research presented in this thesis. We draw overall conclusions on the use of yeast as a cellular model to study the interplay between various phase I and phase II drug metabolizing enzymes. Furthermore the implications of the results from our extracellular biotransformation studies is discussed and future prospects are proposed.
Chapter 1

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